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Characterization of *Azorhizobium caulinodans glnB* and *glnA* Genes: Involvement of the P_{II} Protein in Symbiotic Nitrogen Fixation

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The nucleotide sequence and transcriptional organization of *Azorhizobium caulinodans* ORS571 *glnA*, the structural gene for glutamine synthetase (GS), and *glnB*, the structural gene for the P_{II} protein, have been determined. *glnB* and *glnA* are organized as a single operon transcribed from the same start site, under conditions of both nitrogen limitation and nitrogen excess. This start site may be used by two different promoters since the expression of a *glnB-lacZ* fusion was high in the presence of ammonia and enhanced under conditions of nitrogen limitation in the wild-type strain. The increase was not observed in *rpoN* or *ntrC* mutants. In addition, this fusion was overexpressed under both growth conditions, in the *glnB* mutant strain, suggesting that P_{II} negatively regulates its own expression. A DNA motif, similar to a σ^{54}-dependent promoter consensus, was found in the 5′ nontranscribed region. Thus, the *glnBA* operon seems to be transcribed from a σ^{54}-dependent promoter that operates under conditions of nitrogen limitation and from another uncharacterized promoter in the presence of ammonia. Both *glnB* and *glnBA* mutant strains derepress their nitrogenase in the free-living state, but only the *glnBA* mutant, auxotrophic for glutamine, does not utilize molecular nitrogen for growth. The level of GS adenylylation is not affected in the *glnB* mutant as compared to that in the wild type. Under symbiotic conditions, the *glnB* and *glnBA* mutant strains induced Fix^- nodules on *Sesbania rostrata* roots. P_{II} is the first example in *A. caulinodans* of a protein required for symbiotic nitrogen fixation but dispensable in bacteria growing in the free-living state.

Azorhizobium caulinodans ORS571, isolated from stem nodules of its host plant, the tropical legume *Sesbania rostrata*, fixes nitrogen both during symbiosis and in the free-living state (15). In the free-living state, this strain assimilates fixed ammonium for growth, via the glutamine synthetase (GS)/glutamate synthetase pathway (14). During symbiosis, ammonium produced by nitrogen fixation is exported from the bacteroid to the vegetal cell, where it is assimilated by the plant GS. As in enteric bacteria, only one GS (GSI) has been characterized in *A. caulinodans* (14), whereas two forms (GSI and GSII) have been identified in most rhizobia (9). In enteric bacteria and rhizobia, GS activity is modulated by reversible adenylylation in response to changes in the intracellular glutamine/2-ketoglutarate ratio, reflecting the level of cellular nitrogen (20). In *Escherichia coli*, under conditions of nitrogen limitation, the P_{II} protein, encoded by *glnB*, is uridylylated by the *glnD* gene product. Under conditions of nitrogen excess, P_{II} is deuridylylated and activates an adenylyltransferase (28). This enzyme transfers an AMP group to a tyrosine residue in each of the 12 subunits of the GS; the fully adenylylated form of the enzyme is less active (33). The structural gene for GS (*glnA*) is part of the *glnALG* operon, where *glnL* and *glnG* (also designated *ntrB* and *ntrC*) are the structural genes for NtrB and NtrC, respectively. The transcription of this operon in response to nitrogen availability is under the control of NtrBC. In the presence of P_{II}-UMP, NtrB catalyzes the phosphorylation of NtrC, and NtrC-P activates transcription of the *glnAntrBC* operon at a σ^{54}-dependent promoter (31). Thus, it appears that P_{II} is a major regulatory protein controlling ammonium assimilation in enteric bacteria. It binds directly to 2-ketoglutarate (22) and may also act as a sensor of the available cellular carbon source. Indeed, in a *Synechococcus* sp., P_{II} mediates the coordination of nitrogen fixation and carbon assimilation. A P_{II} mutant has a reduced GS level, is deficient in coupling between photosynthetic nitrate reduction and CO_2 fixation, and is impaired in regulation of methylammonium uptake (17).

In all the proteobacteria of the α subgroup studied, *glnB* and *glnA* are clustered (1a, 11, 21, 25). In *Rhizobium leguminosarum*, *glnB* mutants induce Fix^+ nodules and express *glnA* constitutively, and, as in *E. coli glnB* mutants, the level of GS adenylylation is low (1a). In *Azospirillum brasilense*, *glnB* mutants are unable to fix nitrogen. This Nif^- phenotype is explained by the absence of an active NifA protein (5, 27).

The functional organization of the *glnB* and *glnA* genes of *A. caulinodans* is reported in this article. Since *A. caulinodans* fixes nitrogen in the free-living state and symbiotically, we address the question of the role of the *glnB* gene in both conditions. We report that P_{II} is required for symbiotic nitrogen fixation but is not required in bacteria growing in the free-living state.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, nitrogenase, and β-galactosidase activity assays. The bacterial strains and plasmids used are listed in Table 1. Minimal nitrogen-free medium LSO and growth conditions for *A. caulinodans* were as described previously (15). Nitrogenase assays of bacteria in the free-living state and in planta were performed as reported previously (13, 15). β-Galactosidase assays of bacteria in the free-living state were performed principally as described previously (24). Precultures in the stationary phase were used to inoculate rich medium. The cultures were then harvested in the exponential phase, washed in minimal medium, and inoculated at the same optical density in

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant phenotype[a]	Reference or source
Strains		
A. caulinodans		
ORS571	Wild type	14a
5793	Nif$^-$ Fix$^-$, EMS mutant	36
5721	Nif$^-$ Fix$^-$, EMS mutant; *rpoN*	This laboratory
571C6	Nif$^-$ Fix$^-$ Km^r *ntrC*::Tn*5*	32a
57619	Nif$^+$ Fix$^-$ Km^r Gln$^-$ *glnBA* (*glnB* mutant polar on *glnA*)	This work
57620	Nif$^+$ Fix$^-$ Km^r *glnB*	This work
E. coli S17-1	Sm^r Tp^r Tra$^+$ *pro thi hsdR recA*; RP4 integrated into chromosome; Tc::Mu Km::Tn*7*	33a
Plasmids		
pLA29-17	IncP Tra$^-$ Tc^r Km^r	1
pMTL22$^+$	Amp^r *lacZ′*; pBR322 derivative	8a
pUC4Kixx	Amp^r Km^r (source of *aphII* gene)	Pharmacia
pPHU281	Tc^r *lacZ′ mob* (RP4)	19a
pHP45Ω	Amp^r Sp^r Sm^r (carrying Ω cartridge)	32b
pKOK5	Amp^r Km^r pSUP202 derivative (source of *lacZ-aphII* cartridge)	24a
pRS1023	pLA29-17 carrying the 1.9-kb *Bgl*II fragment encoding the *glnA* gene inserted in the opposite orientation to the *aphII* gene	This work
pRS1024	Same construction as pRS1023, but with *glnA* in the same orientation as the *aphII* gene	This work
pRS1025	pMTL22$^+$ carrying the 1.9-kb *Bgl*II fragment containing *glnA*	This work
pRS1026	pLA29-17 carrying the 18-kb *Sau*3A fragment containing *glnBA*	This work
pRS1027	M13mp19 carrying the 1.6-kb *Sma*I fragment from pRS1026	This work
pRS1028	pPHU281 carrying the 6.6-kb *Sac*I fragment from pRS1026	This work
pRS1029	pRS1028 carrying a *Bam*HI-*Bgl*II deletion and an insertion of the 1.9-kb *Bgl*II fragment from pRS1025 at this site	This work
pRS1030	Same construction as pRS1029 with the 1.6-kb *Bam*HI fragment from pUC4Kixx encoding the *aphII* gene, inserted in the opposite orientation to *glnB*	This work
pRS1031	Same construction as pRS1030 with the *aphII* gene in the same orientation as *glnB*	This work
pRS1032	pUC4Kixx carrying the 1.6-kb *Sma*I fragment from pRS1026	This work
pRS1033	pLA29-17 carrying the 1.6-kb *Bam*HI fragment from pRS1032	This work
pRS2002	*nifH-lacZ*; transcriptional fusion in the broad-host-range vector pGD926	28
pRS1034	*glnB-lacZ*; transcriptional fusion; pRS1033 derivative	This work
pRS1035	*glnA-lacZ*; transcriptional fusion; pRS1033 derivative	This work
pRS1036	*glnBΩA-lacZ*; transcriptional fusion; pRS1033 derivative	This work

[a] EMS, ethyl methane sulfonate.

the appropriate medium for the β-galactosidase assays. β-Galactosidase assays on nodules were performed as follows: nodules from four plants were detached, weighed, and crushed in 2 ml of Z buffer without β-mercaptoethanol. The plant β-galactosidase was inactivated by heat treatment at 50°C for 15 min. Cell debris was removed by centrifugation, and β-mercaptoethanol was adjusted to 0.05 M in the supernatant prior to the enzymatic assay. *E. coli* strains were grown in Luria-Bertani broth. Antibiotics were added to the *A. caulinodans* growth medium at final concentrations of 10 μg/ml for tetracycline, 50 μg/ml for gentamicin, and 100 μg/ml for kanamycin and carbenicillin.

Cloning of the *glnA* and *glnB* genes and construction of pRS1029. A plasmid carrying *glnA* was isolated by complementation of a Gln$^-$ auxotrophic strain (strain 5793), with an *A. caulinodans Bgl*II genomic DNA bank inserted in the pLA29-17 vector and maintained in *E. coli* S17-1 (32). A 1.9-kb *Bgl*II fragment carrying *glnA* was cloned into pLA29-17, in either orientation, to give pRS1023 and pRS1024, and into pMTL22$^+$ to give pRS1025 (Table 1; see Fig. 1). The DNA region upstream from *glnA* was then isolated. A gene bank of *Sau*3A partial digests of ORS571 total DNA, inserted into pLA29-17 (23), was screened by in situ colony hybridization with the 1.9-kb *Bgl*II fragment as a probe. A plasmid carrying an 18-kb fragment was isolated and designated pRS1026. It carries a 6.6-kb *Sac*I fragment and a 1.7-kb *Sma*I fragment overlapping the *glnB* and *glnA* genes. A plasmid carrying the entire *glnB* and *glnA* coding sequences, pRS1029, was then constructed as follows. The 6.6-kb *Sac*I fragment from pRS1026 was inserted into pPHU281, giving pRS1028. The *Bgl*II-*Sac*I fragment from pRS1028, carrying a part of *glnA*, was then excised as a *Bgl*II-*Bam*HI fragment and replaced by the 1.9-kb *Bgl*II fragment from pRS1025 carrying the entire *glnA* coding sequence (see Fig. 1).

DNA sequencing. The nucleotide sequences of both strands of the fragments inserted into pRS1025 (Table 1; Fig. 1) and pRS1027 (Table 1) were determined with the Taquence kit (U.S. Biochemicals). Data were compiled and analyzed with the Genetics Computer Group program. Similarity searches were made with the BLAST program of the National Center of Biotechnology Information server.

RNA preparation and Northern (RNA) blotting. RNA was isolated either from *A. caulinodans* cells grown in medium LSO under microaerobiosis (97% N_2–3% O_2) or from cells grown under aerobiosis in medium LSO containing 20 mM ammonia to an optical density at 600 nm of 0.5. RNA was extracted with hot phenol as described by Gubler and Hennecke (18). Northern blotting was performed with 10 μg of RNA, separated on a 1.2% formaldehyde–agarose gel and transferred to a Hybond-N membrane (Amersham) as described by the manufacturer. Membranes were hybridized with the following two probes: (i) the internal 0.96-kb *Xho*I fragment of *glnA* purified from pRS1025 (Fig. 1) and (ii) the 0.56-kb *Sma*I-*Bgl*II fragment carrying *glnB* (Fig. 1) purified from pRS1032 (Table 1). The probes were labeled with [α-^{32}P]dCTP by use of the random primer labeling kit from Amersham. Hybridizations were for 2 h at 68°C in the Rapid-hyb buffer from Amersham.

Primer extension. Primer extension was performed as described by Ausubel et al. (6) with the following primers labeled with [γ-^{32}P]ATP (see Fig. 2): GlnB1, 5′-CTTGCAGGCCGACTTCTTGAAGGGCCTCC-3′; GlnB2, 5′-GACCGAA CCCCTTTGCCTCGGTGACCGTG-3′; GlnA, 5′-CTTGAT GAAGTCGAGG ACTTCCTTGGCCG-3′. Each Gln primer was mixed with 20 μg of RNA in the hybridization buffer, heated for 10 min at 85°C, and hybridized overnight at 30°C. Primer extension was performed for 90 min at 42°C with 50 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim). The products were separated on a sequencing gel beside a sequencing reaction by using the same oligonucleotide as the primer.

Construction of the mutant strains. The *glnB* mutant strains 57619 and 57620 were constructed by recombination of mutated *glnB* genes carried by pRS1030 and pRS1031, respectively, in the wild-type host genome (Fig. 1). These two plasmids were derived from pRS1029, which is derived from the suicide vector pPHU281. They encode the kanamycin resistance gene (*aphII*), which was purified from pUC4Kixx and inserted at the unique *Bgl*II site of pRS1029 in either orientation. The plasmids were introduced into ORS571 by conjugation, and *glnB* mutants were isolated as kanamycin-resistant, tetracycline-sensitive colo-

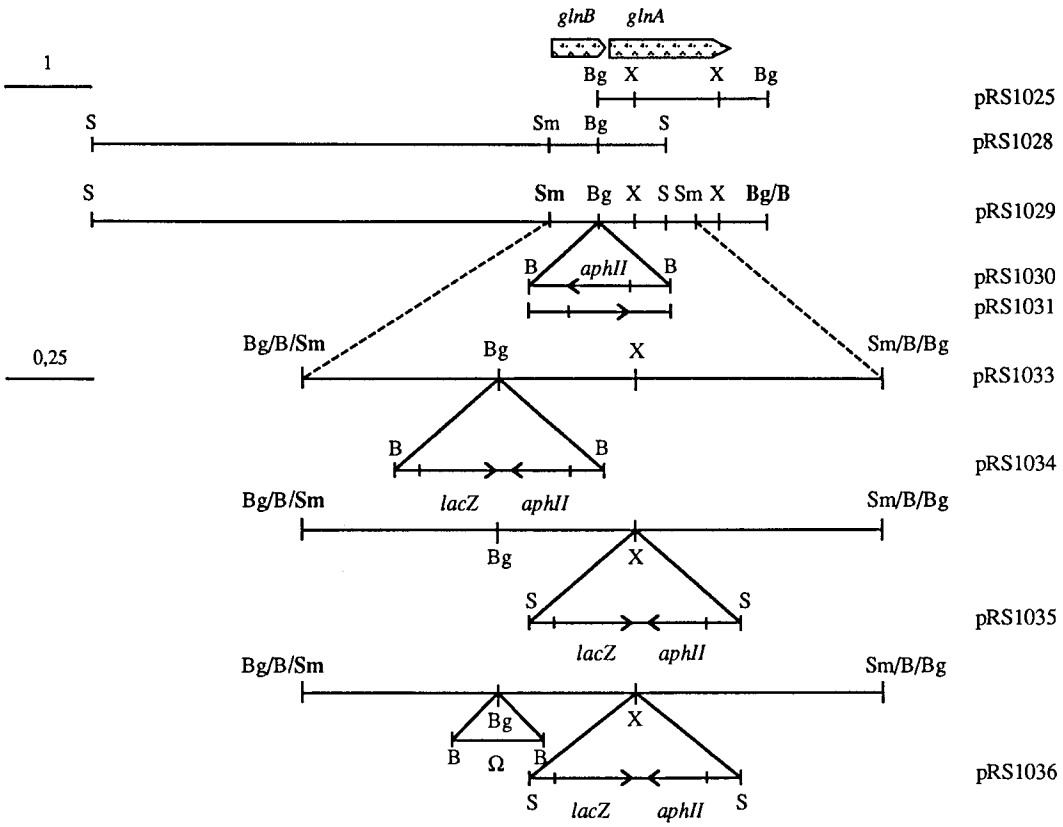

FIG. 1. Physical map of the *A. caulinodans glnBA* region. The positions of *glnB* and *glnA* are indicated by arrows. Vectors are not shown. Construction of *glnB* mutant strains and fusions is shown. Restriction endonuclease site abbreviations: B, *Bam*HI; Bg, *Bgl*II; S, *Sac*I; Sm, *Sma*I; X, *Xho*I. Boldface type indicates the ends of the fragment whose nucleotide sequence was determined.

nies. Southern blotting was performed to verify the correct recombination of the fragment carrying the *aphII* gene.

Construction of the *lacZ* fusions. The pUC4Kixx *aphII* gene was replaced by the pRS1026 1.6-kb *Sma*I fragment to give pRS1032. The plasmid was then cleaved with *Bam*HI and ligated to *Bgl*II-cleaved pLA29-17, to give pRS1033. pRS1034, carrying the *glnB-lacZ* fusion, was constructed by inserting the *lacZ-aphII* cartridge, purified as a 4.7-kb *Bam*HI fragment from pKOK5, at the *Bgl*II site of pRS1033. pRS1035, carrying the *glnA-lacZ* fusion, was constructed by inserting the same cartridge, purified as a *Sal*I fragment, at the *Xho*I site of pRS1033. pRS1036, carrying the *glnB*Ω*A-lacZ* fusion, was constructed by inserting the 2-kb *Bam*HI fragment from pHP45Ω, which carries the streptomycin-spectinomycin resistance gene and contains transcription termination sequences at both ends, at the *Bgl*II site of pRS1035 (Fig. 1).

GS assays. Cultures grown in rich medium to an optical density at 600 nm of 0.25 were pelleted, washed, resuspended in 30 ml of minimal medium LSO, and incubated overnight at 30°C under microaerobic conditions (97% N_2–3% O_2). An ammonia shock was then given by injecting ammonia at a final concentration of 0.2% into the flasks and incubating the cultures for a further 30 min. Whole cells were then assayed for GS activity by use of the transferase reaction described by Donald and Ludwig (14). The assay was also performed on control cells that had not received the ammonia shock.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been assigned the accession number Y10213 in the EMBL Nucleotide Sequence Database.

RESULTS

Cloning and sequencing of the *glnBA* genes. It was previously established (32) that the mutant strain of *A. caulinodans* 5793, induced by ethyl methanesulfonate, is a glutamine auxotroph. Prototrophy was restored by pRS1024 carrying a 1.9-kb *Bgl*II fragment. This fragment hybridizes with a DNA probe carrying the *Azospirillum brasilense glnA* gene (data not shown), suggesting that it carries the *A. caulinodans glnA* gene. Complementation of strain 5793 was not observed when the fragment was cloned in the opposite orientation (pRS1023), suggesting that *glnA* is transcribed from the pRS1024 *aphII* promoter. The 1.9-kb *Bgl*II fragment was then used as a probe to isolate pRS1026, which carries a much larger insert and restores prototrophy to the mutant strain 5793. The physical map of the pRS1026 insert is the same as that of genomic *glnA* as determined by hybridization experiments. The nucleotide sequence of the 2.45-kb fragment from the first *Sma*I site to the second *Bgl*II site in pRS1029 (Fig. 1) was determined. There are two open reading frames, 53 bp apart. The first encodes a 112-amino-acid polypeptide with 82% identity with the P_{II} protein of *A. brasilense* (11). The second encodes a 468-amino-acid polypeptide, with 71% identity with the GSI of *Rhizobium leguminosarum* (16). The nucleotide sequences of *glnB* and the flanking regions are given in Fig. 2. The Tyr residue, at position 51 of the *A. caulinodans* P_{II} protein, corresponding to the uridylylation site in the *E. coli* protein (34), is conserved, suggesting a possible modification of the protein by uridylylation. A putative *glnB* promoter sequence, similar to a σ^{54}-dependent promoter consensus, TGGCA-N_6-GTGCTT (7a), was identified upstream from *glnB.*

Analysis of *glnBA* transcription. The transcriptional organization of the *glnB* and *glnA* genes was studied by Northern blotting to determine the size and relative abundance of transcripts. RNA was extracted from nitrogen-fixing bacteria and from cultures grown in the presence of ammonia and hybridized with a *glnB* probe (Fig. 3A) or with a *glnA* probe (Fig. 3B). Transcripts of 2.2 and 1.8 kb were detected with the *glnA* probe

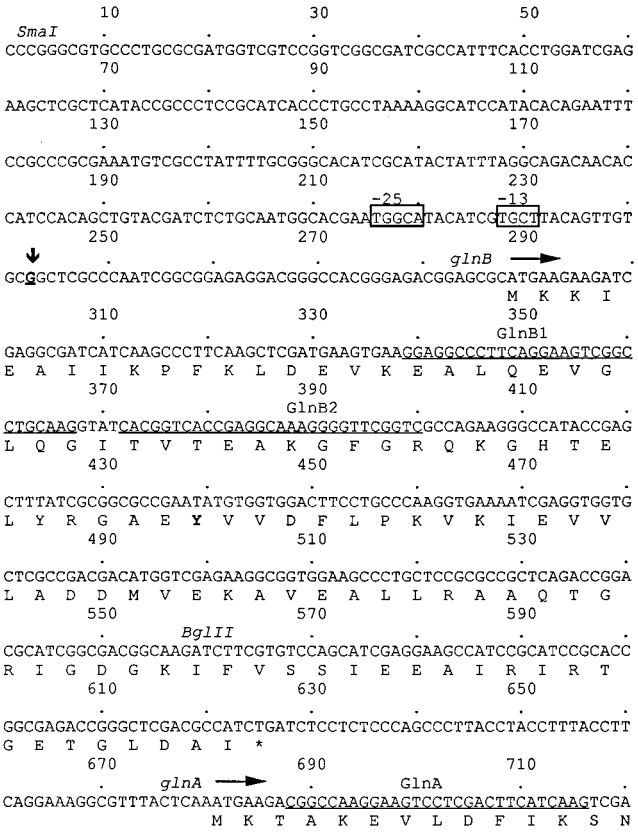

FIG. 2. Nucleotide and deduced amino acid sequences of *A. caulinodans glnB* and flanking regions. The ATG initiation codon of *glnB* is in position 289. The ATG initiation codon of *glnA* is in position 681. Horizontal arrows indicate the positions of the genes. Regions complementary to the oligonucleotides used are underlined. A vertical arrow indicates the transcription start site. The σ^{54} consensus sequence is boxed. Relevant restriction sites are indicated in italics. The conserved Tyr in position 51 is indicated in boldface type.

in the wild-type strain. The 2.2-kb transcript was also detected with the *glnB* probe, suggesting that it is the *glnBA* transcript. The 1.8-kb transcript was not detected with the *glnB* probe, suggesting that it corresponded to a *glnA* transcript. This was investigated further with RNA preparations from the mutant strains 57619 and 57620 carrying an *aphII* gene in the *glnB* coding sequence. Strain 57620 carries the 1.6-kb *aphII* gene in the same transcriptional orientation as *glnB*. Disruption of the gene caused an increase in the relative sizes of transcripts detected with the *glnA* probe. Three transcripts from strain 57620 of 3.7, 2.8, and 1.8 kb were detected (Fig. 3B). The 3.7-kb transcript is probably *glnBA* plus the *aphII* transcripts initiated from the *glnB* promoter. The 2.8-kb transcript is probably initiated from the *aphII* promoter (*aphII glnA* transcript), and the 1.8-kb transcript is thought to be the *glnA* transcript. In strain 57619, which carries the *aphII* gene in the transcriptional orientation opposite to that of *glnB*, these three transcripts were not detected with the *glnA* probe (Fig. 3A), suggesting that *glnA* is not transcribed from a promoter between *glnB* and *glnA*. Both *glnB* and *glnA* were transcribed in the presence or absence of ammonia (Fig. 3). The *glnA* transcript from ORS571 and 57620 was more abundant than the other transcripts (Fig. 3B), presumably due to processing of the *glnBA* or the *aphII glnA* transcripts. This transcript is much more abundant in strain 57620 than in the wild-type strain, indicating that the *aphII* promoter is more active than the promoter upstream from *glnB*.

The transcriptional start site of the *glnB* and *glnA* genes was mapped by primer extension experiments with three primers, namely, GlnB1, GlnB2, and GlnA (Fig. 2). RNA was extracted from a wild-type *A. caulinodans* culture, from a culture of the same strain carrying pRS1026 to enhance the signal, and from an *rpoN* mutant (strain 5721). A signal was reproducibly detected at 46 nucleotides upstream from the *glnB* start codon with the GlnB2 primer (Fig. 4A) and the GlnB1 primer (Fig. 4B), with RNA from cultures grown in the presence of glutamine (Fig. 4A, lane 1) or ammonia (Fig. 4B, lanes 1 and 3) or under nitrogen-fixing conditions (Fig. 4B, lane 2). This signal is located 13 bp downstream from the σ^{54} consensus sequence. It was detected with RNA from the wild-type strain regardless of whether it harbored pRS1026. It was also detected with RNA from the *rpoN* mutant strain 5721 in either the presence or absence of ammonia (data not shown). This suggests the existence of two overlapping promoters with the same start site, one of them σ^{54} dependent and the other uncharacterized. Several signals were detected with the GlnA primer (data not shown). The major one is similar to that detected with GlnB1 and GlnB2 primers, indicating that *glnB* and *glnA* are transcribed from the same start site. The other signals, which were weak, were located between *glnB* and *glnA* and could explain the presence, on the Northern blot, of a smaller transcript (Fig. 3B).

Expression of *glnB-lacZ*, *glnA-lacZ*, and *glnBΩA-lacZ* fusions. Different *lacZ* fusions were constructed to determine the role of the σ^{54} promoter-like sequence upstream from *glnB* in transcription. In pRS1034, the *lacZ-aphII* cartridge is inserted in the *glnB* coding sequence (*glnB-lacZ*), and in pRS1035, the same cartridge is inserted in the *glnA* coding sequence (*glnA-*

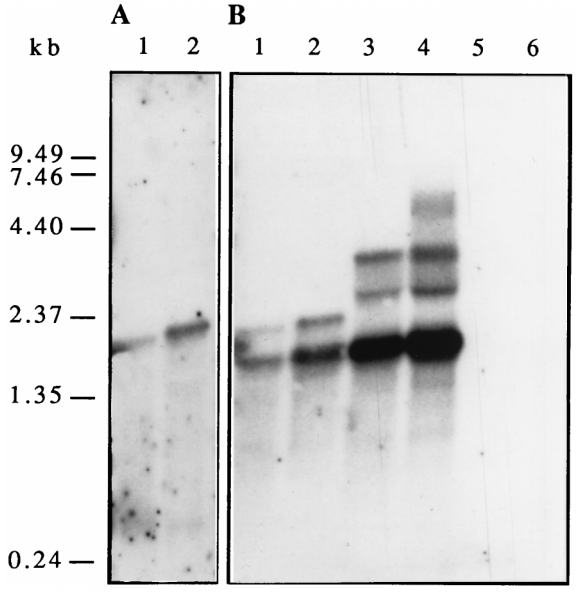

FIG. 3. Northern blotting of *glnB*-specific (A) and *glnA*-specific (B) transcripts from *A. caulinodans* strains grown either in minimal medium with 97% N_2–3% O_2 (LSO–3% O_2) or in minimal medium supplemented with 20 mM NH_4^+ in air (LSN). The same Northern blot was probed sequentially with the two gene-specific probes, as described in Materials and Methods. Lanes: 1 and 2, RNA from ORS571 grown in LSO–3% O_2 (lane 1) or LSN (lane 2); 3 and 4, RNA from 57620 grown in LSO–3% O_2 (lane 3) or LSN (lane 4); 5 and 6, RNA from 57619 grown in LSO–3% O_2 (lane 5) or LSN (lane 6). A molecular size ladder of RNA is shown on the left.

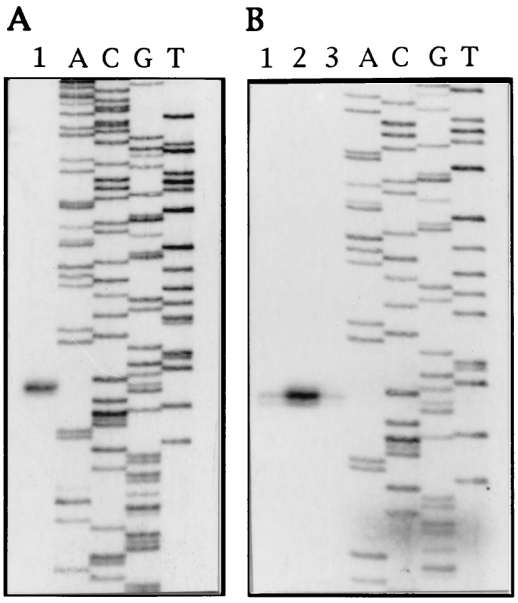

FIG. 4. Mapping of the transcription start site of the *glnBA* mRNA from the wild-type strain and from the same strain carrying pRS1026. (A) Extension with primer GlnB2. Lane 1 contains mRNA from ORS571 grown in minimal medium with 0.5 mg of glutamine per ml. (B) Extension with GlnB1. Lanes: 1, mRNA from ORS571pRS1026 grown in minimal medium with 20 mM NH_4^+; 2, mRNA from the same strain grown in minimal medium with 97% N_2–3% O_2; 3, mRNA from ORS571 grown in minimal medium with 20 mM NH_4^+.

lacZ) (see Materials and Methods). These fusions were introduced into the wild-type strain and into mutants 571C6 (*ntrC*), 5721 (*rpoN*), and 57620 (*glnB*), and their expression was measured after 2, 3, or 4 h of incubation under conditions of nitrogen fixation (Fig. 5A) or ammonia assimilation (Fig. 5B). The *glnB-lacZ* fusion was expressed at similar levels (about 3×10^4 Miller units · mg of protein^{-1}), under conditions of ammonia assimilation, in the wild-type strain and in the *rpoN* and the *ntrC* mutants (Fig. 5B). Under conditions of nitrogen fixation, *glnB-lacZ* expression in the *rpoN* and *ntrC* mutants was unchanged, whereas it was increased 1.5-fold in the wild-type strain (Fig. 5A). The high level of expression of this fusion in the wild-type and mutant strains may be due to the presence of a constitutive promoter, functioning under conditions of excess ammonia. The 50% higher expression in the wild-type strain with respect to that in the mutants, under conditions of nitrogen limitation, is probably due to transcription from a σ^{54}-dependent promoter controlled by NtrC. In strain 57620 (*glnB*), the level of expression of the *glnB* fusion was twofold higher than that in the wild-type strain under both growth conditions. This suggests a negative retrocontrol of P_{II} on its own synthesis. Similar results were obtained with the *glnA-lacZ* fusion. To verify the absence of a promoter between *glnB* and *glnA*, we have inserted an interposon in the *glnB* coding sequence of the *glnA-lacZ* fusion (pRS1036; *glnBΩA-lacZ*). β-Galactosidase activity was not detected above the background level with the *glnBΩA-lacZ* fusion (data not shown), confirming that *glnA* is transcribed from the same promoter as *glnB*.

Phenotype of *glnB* mutant strains. A physiological analysis of strains 57619 (*glnBA*) and 57620 (*glnB*) was undertaken to determine the role of the P_{II} protein in *A. caulinodans*. As expected, strain 57619, which is a glutamine auxotroph, did not grow with ammonia as a sole nitrogen source. Strain 57620 was prototrophic, and although the maximal optical density in minimal ammonia medium culture was identical to that of the wild type, its growth rate was slightly reduced.

(i) The *glnB* mutation has a moderate effect on GS adenylylation. *A. caulinodans* GS activity is modulated by the adenylylation-deadenylylation process as reported by Donald and Ludwig (14). GS activity was measured to determine whether the *A. caulinodans* P_{II} protein is involved in this process as documented for *E. coli*. Total GS activity was measured by the γ-glutamyltransferase assay in the absence of Mg^{2+}, and the activity of the deadenylylated (active) form of the enzyme was measured by the same assay in the presence of 60 mM Mg^{2+}. A higher percentage of active GS is present under conditions

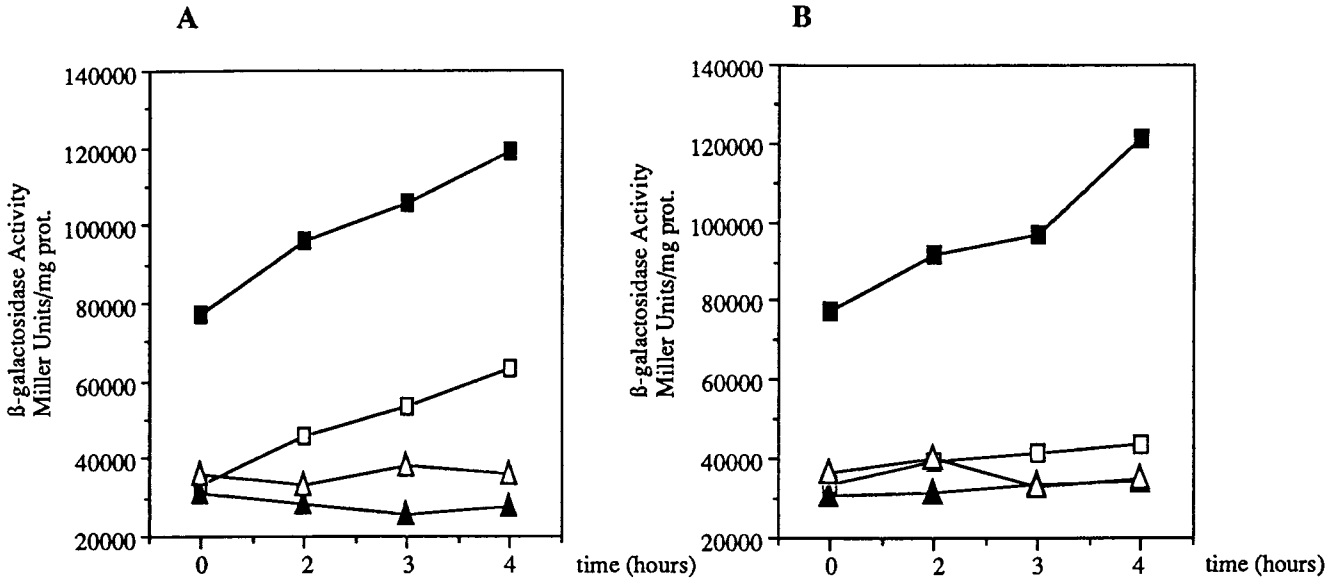

FIG. 5. β-Galactosidase activity of *glnB-lacZ* fusions expressed in Miller units per milligram of protein. (A) Condition of nitrogen fixation; (B) condition of ammonia assimilation. Symbols: □, strain ORS571 (wild type); ■, strain 57620 (*glnB*); △, strain 5721 (*rpoN*); ▲, strain 571C6 (*ntrC*).

TABLE 2. GS specific activity in *A. caulinodans* ORS571 and 57620 (*glnB*) strains grown under different physiological conditions

Strain	GS sp act[a,b]		% Unadenylylated GS[b]	
	$-NH_4^{+c}$	$+NH_4^{+d}$	$-NH_4^+$	$+NH_4^+$
571	1.77 ± 0.11	1.02 ± 0.02	36.0 ± 12.0	10.6 ± 6.6
57620	8.36 ± 0.34	4.97 ± 0.61	32.6 ± 5.1	13.5 ± 1.9

[a] Specific activity of GS in pure culture; 1 unit corresponds to 1 μmol of γ-glutamyl hydroxamate · min^{-1} · mg of $protein^{-1}$.
[b] Values are the means ± standard deviations from at least three independent experiments.
[c] Cultures were grown overnight in minimal medium devoid of ammonia with 97% N_2–3% O_2.
[d] Cultures were grown overnight in minimal medium with 97% N_2–3% O_2 and shocked for 30 min by the addition of 0.2% ammonia.

of nitrogen fixation than under ammonia assimilation in both the wild-type and *glnB* mutant (57620) strains (Table 2). However, the levels of unadenylylated GS were the same in 57620 and in the wild-type strain when cultures were grown under conditions of ammonia excess. Therefore, P_{II} is not involved in GS adenylylation. Total GS activity is fivefold higher in strain 57620 than in the wild-type strain (Table 2), due to the higher level of transcription of *glnA* (Fig. 3).

(ii) P_{II} is not required for nitrogen fixation in the free-living state. Both the 57619 and 57620 mutants derepress their nitrogenase activity in the free-living state (Table 3). Mutant strain 57620 can grow in nitrogen-free solid medium at the expense of molecular nitrogen, whereas strain 57619, which is auxotrophic for glutamine, cannot. Therefore P_{II} is not required for nitrogen fixation in pure culture in *A. caulinodans.*

(iii) P_{II} is essential for symbiotic nitrogen fixation. Inoculation of *S. rostrata* roots with *glnB* (strain 57620) or *glnBA* (strain 57619) mutants showed that both strains affected plant development. After 5 weeks, plants inoculated with the mutant strains were the same size as those inoculated with the wild-type strain, but the leaves were small and yellow and there were half the number of leaves in the mutant-inoculated plants as compared with the plants inoculated with the wild type. Although these strains formed as many nodules as the wild type, nitrogenase activity was not detected (Table 3). This was not due to lack of infection of the nodule tissue or to lack of *nifH* transcription since a *nifH-lacZ* fusion was expressed at similar levels in nodules of plants inoculated with 57620 or with ORS571 (about 2×10^3 Miller units · mg of $protein^{-1}$). Therefore, strain 57620 did not establish the physiological conditions necessary for nitrogenase activity.

DISCUSSION

We report on the transcriptional organization of the *glnB* and *glnA* genes and the phenotypic characterization of two *glnB* mutants. *glnB* and *glnA* are organized as a single operon transcribed from the same start site upstream from *glnB* as determined by *lacZ* fusion experiments and primer extension analysis. The *glnA* transcript is more abundant than the *glnBA* transcript as shown by Northern blotting, suggesting a post-transcriptional processing event, as reported for the regulation of the *glnBA* operons of *Rhodobacter capsulatus* (8) and *Rhodospirillum rubrum* (21). In *Rhodobacter capsulatus*, a putative stem-loop structure at the 3′ end of the *glnB* transcript may be the processing site of an endoribonuclease, allowing rapid turnover of the upstream mRNA. This mechanism has also been proposed to explain the differential stability of the *fixABCX* transcripts in *A. caulinodans* (4).

Expression of *glnBA* was high under conditions of nitrogen excess and further enhanced under conditions of nitrogen limitation, as shown by *lacZ* fusions in the wild-type strain. Furthermore, this high level of expression in the presence of ammonia is observed in *rpoN* or *ntrC* mutant strains. On the other hand, the increase of expression under conditions of nitrogen limitation was not observed in *rpoN* or *ntrC* mutant strains, suggesting that the operon is transcribed from two promoters, the σ^{54}-dependent one activated by NtrC under conditions of nitrogen limitation and the constitutive one operating under nitrogen excess. This regulatory mechanism would provide a constant level of P_{II} and GS, although it would be higher under nitrogen-limiting conditions. Such a mechanism has been reported for other *glnBA* operons such as that of *Bradyrhizobium japonicum* (29), in which the two promoters are far apart and initiate transcription from two independent start sites. In *A. caulinodans*, only one *glnBA* transcriptional start site was detected in primer extension experiments, suggesting that the same site is shared by both promoters. This is relatively unusual but has been reported for the *nifA* gene of *Bradyrhizobium japonicum* (7), where the *fixRnifA* operon is transcribed from two overlapping promoters, recognized by different RNA polymerase holoenzymes, $E\sigma^{54}$ and $E\sigma^{96}$, that initiate transcription at the same site.

In contrast to the Nif^- phenotype of the *glnB* mutant of *Azospirillum brasilense*, the *glnB* mutant of *A. caulinodans* fixes nitrogen in the free-living state like the wild type does. This suggests that P_{II}, the product of *glnB*, has no effect on free-living nitrogen fixation, as is the case for *Klebsiella pneumoniae* (19) and *Rhodobacter capsulatus* (25).

GS specific activity assays showed that P_{II} plays no role in GS adenylylation. This suggested the presence of a second P_{II} protein as described for *E. coli* (36) and *Azospirillum brasilense* (12). Indeed, a second copy of *glnB*, designated *glnZ*, was located upstream from a *nrgA*-like gene (unpublished results), as reported for *Bacillus subtilis* (37). It encodes a polypeptide similar to P_{II} of *A. caulinodans*, GlnK of *E. coli*, and P_Z of *Azospirillum brasilense. glnZ* is, like *glnB*, transcribed from a σ^{54}-dependent promoter activated by NtrC. In contrast to *glnB*, *glnZ* is not expressed in the presence of ammonia or in the *rpoN* mutant strain, which is another indication that *glnB* is transcribed from two different promoters (unpublished results). The characterization of a double P_{II}-P_Z-like mutant is under investigation and will allow us to determine if a complex of the two P_{II} proteins of *A. caulinodans* is involved in GS adenylylation.

Phenotypic analyses of *glnB* mutants in the host plant showed that whereas the mutation does not affect nitrogenase activity in the free-living state, both *glnB* and *glnBA* mutants

TABLE 3. Nitrogenase specific activity in pure culture and in nodules of 5-week-old plants inoculated with *A. caulinodans* ORS571, 57619 (*glnBA*), or 57620 (*glnB*)

Strain	Nitrogenase sp act[a]		Relevant phenotype[b]
	In the free-living state[c]	In planta[d]	
571	28.9 ± 3.4	0.14 ± 0.06	Nif^+ Fix^+
57619	21.1 ± 2.6	0.01 ± 0.01	Nif^+ Fix^-
57620	22.3 ± 3.9	0.03 ± 0.02	Nif^+ Fix^-

[a] Data are the means ± standard deviations from at least three independent experiments.
[b] Nif^+ refers to the capacity to derepress nitrogenase activity in the free-living state; Fix^+ refers to the same capacity during symbiosis.
[c] Expressed in nanomoles of ethylene per minute per milligram of protein.
[d] Expressed in nanomoles of ethylene per minute per milligram of nodules.

are Fix$^-$ in planta. This strongly supports the assumption that P_{II} is necessary for symbiotic nitrogen fixation. In previous works, it has been suggested that *glnA* was required only for the establishment of functional bacteroids. This was based on the fact that GS activity was not detected in mature bacteroids, while Gln$^-$ strains displayed a Fix$^-$ phenotype (10, 14). In the present work, whether GS is required for symbiotic nitrogen fixation in addition to P_{II} cannot be elucidated. Since *glnA* is transcribed from the *glnB* promoter, it is possible that in the wild-type bacteroid, GS synthesis occurs. This would be in agreement with the low level of partially active GSI detected in both *Rhizobium meliloti* and *Rhizobium etli* bacteroids (2, 30).

The in planta Fix$^-$ phenotype of the *glnB* mutant was unexpected since *A. caulinodans* mutants generally display the same phenotype in the free-living state and in the symbiotic state. This phenotype appears to be specific to *glnB* since a *glnZ* mutant is Nif$^+$ Fix$^+$ (unpublished results). Several hypotheses can be proposed to explain the Fix$^-$ phenotype. The absence of P_{II} might affect carbon metabolism and hence the synthesis of adequate ATP for nitrogenase activity. Alternatively, P_{II} may be involved in ammonium transport from the bacteroid to the vegetal cell. Indeed, in *B. subtilis*, *nrgB*, which encodes a P_{II} homolog, is linked to *nrgA*, which is thought to encode an extracellular sensor of nitrogen (37). This transcriptional organization suggests that the two proteins have related functions. The P_{II} protein of *Rhizobium meliloti* is also thought to be involved in ammonium transport in the nodule, based on experiments with alfalfa plants which appeared to be nitrogen starved when inoculated with a *glnB* mutant, whereas the nodules exhibited wild-type nitrogenase activity (3). Considering the data obtained with *A. caulinodans*, and given the existence of a regulatory mechanism controlling the activity of the nitrogenase (26), a deficiency in ammonium transport could explain both the poor development of the plant and the inactivation of nitrogenase and thus the Fix$^-$ phenotype of a *glnB* mutant strain. Channel-like NH_4^+ transporters have recently been reported on the surface of *Bradyrhizobium japonicum* symbiosomes (35). Further work is necessary to determine whether such transporters exist in *A. caulinodans* bacteroids and, if so, whether they are regulated by P_{II}.

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